MODEL FOR TESTING IMMUNOGENICITY OF PEPTIDES

Government Interest

The invention described herein may be manufactured, licensed and used by or for governmental purposes without the payment of any royalties to us thereon.

Cross Reference

This application is a continuation in part of U.S. Patent Application Serial No. 08/789,734 filed January 27, ~~1998~~ 1997 which in turn is a continuation in part of U.S. Patent Application Serial No. 08/590,973 filed January 24, 1996 which in turn is a continuation-in-part of U.S. Patent Application Serial No. 08/247,884 filed May 23, 1994 which in turn is a continuation-in-part of U.S. Patent Application Serial No. 08/064,559, filed ~~May 21, 1993~~ and the present application incorporates U.S. Patent Application Serial Nos. 08/064,559, 08/789,734, 08/590,973 and 08/247,884 in their entirety by reference.

Field of the Invention

This invention relates to a means of predicting potential of a peptide for eliciting immune response.

Background of the Invention:

Among the numerous steps required for an immunological response to occur is the presentation of the antigen by macrophages to the B-cell or T-cell. This presentation is mediated by the Class I and Class II major histocompatibility complex (MHC) molecules on the surface of the cell. The MHC molecules hold antigens in the form of the peptide fragments and together with the receptor molecule on the T-cells,

form a macromolecular complex that induces a response in the T-cell. Therefore, a necessary step in an immune response is the binding of the antigen to the MHC.

Recent single crystal X-ray structures of human and murine Class I MHC's have been reported. Analysis of these crystal structures have shown that antigenic peptides lie in the so-called binding cleft for presentation to the T-cell. This cleft is formed by α_1 and α_2 domains and by β-strands from each domain forming the floor. Furthermore, the sequence polymorphism among Class I molecules can result in alterations of the surface of the cleft forming different pockets. Peptide side chains may insert into these pockets. Thus, different pockets may interact with different side chains. This implies the mechanism for the peptide specificity of class I MHC's. Peptides bound to the Class I MHC's in the crystal structures were found to have both the amino and carboxy termini tightly held by the MHC. There were few interactions near the middle of the cleft. Hence the bound peptide is allowed to bend slightly in the center. The observed binding mode helped to explain the apparent partial specificity of peptide sequence and the allowed variation in peptide length found among peptides isolated from Class I MHC's.

The precise mode of binding of peptides to Class II MHC molecules is less clear. While a single crystal X-ray diffraction structure for the HLA-DR1 MHC has been shown, the coordinates have remained unavailable. However, currently available theoretical and experimental results help form a hypothesis that the binding of a peptide to Class II MHC is similar to that

observed with Class I. First, it is noted that the Class II binding cleft is structurally similar to that of Class I. This was concluded based upon a sequence analysis of 26 Class I and 54 Class II amino acid sequences.

Unlike with Class I molecules, self-peptides isolated from murine I-A^b and I-E^b, from murine I-A^d and from human HLA-DR1 molecules were found to be varied in size (13 to 25 residues long). The peptides isolated from the murine I-A^b and I-E^b molecules had heterogenous carboxy termini while those from I-A^d and HLA-DR1 had ragged termini at both ends. The varying lengths indicate that the amino and carboxy termini of the peptides were not critical for the binding. One or both termini may protrude from the binding site and be available for further processing. The residues critical for binding were proposed to be at the ends of the peptide as opposed to the center.

Summary of the Invention:

It is the purpose of this invention to provide a method for preliminary screening of peptides for ability to elicit an immune response. Structural homology techniques were used to model a receptor (the Class II MHC is exemplified). This model makes it possible to preliminarily screen peptides for antigenic properties. By modifying the peptide to "fit" into the receptor it is possible to identify methods of rendering non-immunogenic peptides immunogenic.

The preliminary screening of peptides for immunogenicity comprises the steps of (1) creating a molecular model of a receptor followed by minimizing the model created, 2) modeling a peptide to be tested

and minimizing the model of the peptide, then testing the fit of the model of the peptide into the model of the receptor to produce a composite minimized receptor/minimized peptide model. Upon finding an acceptable fit, the peptide may then be screened by a binding assay for actual binding to Class II MHC as a further test for immunogenicity.

It has been found that when the model of the peptide can not be fitted into the model of the receptor, the peptide will lack immunogenicity. While not all peptide models which can be made to "fit" into the model of the receptor will be effective as immunogens, the screening methods of the invention may make it possible to avoid undue biological testing of inappropriate peptides. By using the model, it is also possible to alter peptides to accommodate the receptor. Hence, the invention has both predictive and drug design applications.

Brief Description of the Figures:

Fig. 1 shows the HLA-aw68 α_1 and α_2 domains with DR1 α_1 and β_1 domains aw68 α_1, and α_2 domains are represented by SEQ ID NO:13, DR1 α, and β_2 domains are represented by SEQ ID NO:14 and SEQ ID NO:15 respectively.

Figs. 2-30 are a printout of the minimized coordinates of the receptor.

Figs. 31 and 32 shows the effects of various peptides inhibiting the binding of labeled hemagglutinin in a competitive binding assay.

Detailed Description of the Invention:

In order to understand and better predict peptide interaction with Class II MHC's and as an aid for

synthetic peptide vaccine design, a structural homology model of HLA-DR1 molecule was made using the Class I HLA-aw68 as a reference molecule. For purposes of this analysis, numerous conserved residues were aligned leading to a proposed three-dimensional model for the Class II structure very similar to that of Class I. This model retained the overall conformation of a Class I MHC and agreed with a considerable amount of the published data. Furthermore, peptides shown to bind to DR1 were docked in the binding cleft of the model and analyzed. The results agree with the experimental binding data presented here. Hence, it is shown that the structural homology model reported here is useful for screening Class II MHC functionality.

It had been hypothesized that few peptide residues may be required for binding to DR1. By substituting residues into the influenza hemagglutinin 307-319 T-cell epitope (HA) it had been determined that a single tyrosine at 308 was required for binding. A synthetic peptide with the tyrosine at position 308 and a lysine at 315 was found to bind DR1 as well as the native peptide. Hence, it was concluded that few peptide residues determine the high affinity binding to DR1.

The peptides produced according to the present invention may be used alone or chemically bound to another peptide and/or carrier in order to elicit an immune response. An immune response is elicited by administering a peptide to an animal in an effective dose and by an effective route of administration. Typically the peptide will be administered with an immunologically acceptable carrier. The routes of administration, dosages, times between multiple administrations will be based on the particular peptide and are standard operations of those skilled in the art.

Of particular interest are peptides from pathogenic microorganisms and neoplasms. In such an example, a vaccine may be formed with the peptide and any known immunological carrier and may be administered prophylactically or therapeutically. The immune response may be elicited for a number of reasons other than for prophylaxis or therapy such as increasing antibody production ~~form~~ from the harvesting of antibodies, or increasing specific B-cell or T-cell concentration for the production of hybridomas or cellular therapy.

The choice of host animals is limited only to those capable of an immune response. Preferred hosts are mammals, more preferred are humans.

The vaccine may contain plural peptides with each peptide corresponding to the same or different antigens. The peptides may be used unbound or they may be chemically bound to another peptide or an unrelated protein or other molecule. A preferred vaccine preparation contains a plurality of peptides chemically bound to a larger more immunogenic peptide.

The peptide or plurality of peptides may be adsorbed, bound or encapsulated in a biodegradable microsphere, microcapsule, larger carrier or a combination of these. The carrier may have a slow or controlled release property thereby releasing the peptide under appropriate conditions and times for enhanced immunization. This is particularly important when administering the peptide orally where stomach acid can degrade the peptide.

When the peptide is combined (i.e. encapsulated within) with a biodegradable lactide and/or glycolide polymers, they can be formulated into immunostimulating composition comprising encapsulating- microspheres, which may contain a pharmaceutically-acceptable adjuvant, wherein said microspheres having a diameter

between 1 nanogram (ng) to 10 microns (um) are comprised of (a) a biodegradable-biocompatible poly (DL-lactide-co-glycolide) as the bulk matrix, wherein the relative ratio between the amount of lactide and glycolide components are within the range of 40:60 to 0:100 and (b) an immunogenic substance comprising Colony Factor Antigen (CFA/II), hepatitis B surface antigen (HBsAg), or a physiologically similar antigen that serves to elicit the production of antibodies in animal subjects. Compositions wherein the immunogenic substance is a peptide within the range of 0.1 to 1.5% based on the volume of the bulk matrix of lactide and glycolide component having a relative ~~ration~~ ratio of 48:52 to 58:42 should be especially useful.

Another embodiment of the present invention is to modify the amino acid sequence of a peptide to enhance its immunogenicity. This is done by modifying the natural peptide sequence to bind to the Class II MHC receptor DR12 with superior binding affinity for a Class II MHC receptor DR1 than the natural peptide sequence. This modified peptide is considered a synthetic peptide. Alternatively, the sequence may be modified to have a greater inhibition of HA (306-318) binding to a Class II MHC receptor DR1.

Many amino acid changes are acceptable in the formation of a synthetic peptide. The changes may be for similar types of amino acids such as leucine for isoleucine or they may be for diverse types such as tyrosine for lysine.

Materials and Methods:

The structural homology model for the DR1 Class II MHC was constructed using the QUANTA molecular modeling package (vision 3.2, Molecular Simulations, Inc., Burlington, MA) with the CHARMM and Protein Design modules. After alignment of the sequences as described

below, gaps and loops were energy minimized using 100 steps of steepest descents minimization followed by 100 steps of adopted basis set Newton-Rapheson (ABNR) minimization. Large gaps were closed using a fragment database from a selected set of high-resolution crystal structures. The resulting structure we minimized in vacuo using 1000 steps of steepest descents followed by an additional 1000 steps of ABNR minimization. A distance related electrostatic function was used in all calculations with a dielectric constant of 1.0. Non-bound parameter lists were updated every 20 steps with a cutoff distance of 15.0A. Non-bonded calculations were performed using a shifted potential function between 11.0A and 14.0A. An extended atom set was used with only polar hydrogen atoms specifically placed. There were no explicit hydrogen bond energy calculations performed.

All peptides were initially modeled using QUANTA in an extended chain conformation and subjected to 500 steps of ABNR minimization. The resulting structures remained essentially in extended chain conformations. Individual peptides were manually docked in several different orientations into the binding cleft region of the minimized DR1 structure. The resulting bimolecular complex was subjected to 5000 steps of steepest descents minimization with non-bonded interactions updated every five steps. After minimization, bound peptides remained essentially in extended chain conformations. The lowest energy complexes for each peptide were selected for further analysis.

The selected peptide and DR1 complexes and the minimized DR1 model were subjected to the following molecular dynamics regimen: 300 steps of heating to 300°K, 600 steps of equilibration at 300°K, and 1100 steps of production dynamics. During this simulation,

the DR1 Cα atoms were constrained in their starting positions. All non-bonded interaction parameters were as stated for the minimization procedure. The lowest energy structure during the course of the production dynamics was selected and subjected to the 5000 step minimization procedure described previously with the Cα restraints removed. The resulting structures were used for the binding energy calculations and for hydrogen bonding analysis.

Hydrogen bonds were determined using the QUANTA default parameters. Maximum allowed distances were 2.5Å between a hydrogen and the acceptor atom and 3.3Å between the donor and acceptor atoms. The minimum angle allowed between any set of atoms forming a hydrogen bond was 90°.

Competitive Inhibition Binding Assay:

HA peptide (the influenza hemagglutinin 307-319 T-cell epitope) was labeled with ^{125}I. The labeled HA peptides were then allowed to interact with purified DR1 molecules during incubation to allow formation of peptide/DR1 complexes. After incubation, the peptide/DR1 composition was exposed to a native gel for chromatographic separation or passed through a spun column to separate labeled peptide/DR1 complex and free labelled peptide. When unlabeled peptides were added before incubation of labeled HA peptides and DR1, and if the unlabelled peptides had capacity for binding to DR1 simultaneous with ^{125}I-HA, there was a resultant decrease in radioactive signal associated with the DR1. The extent of this decrease directly related to the binding capacity of the unlabeled unknown peptide.

Structural Homology Model for the DR1 Molecule:

The structural homology model was created, the reference molecule being the crystal structure of HLA-aw68. The HLA-aw68 coordinates and subsequent sequence were obtained from the entry 2HLA in the Brookhaven Protein Data Bank released January 15, 1991, which is incorporated herein by reference. The sequence for the DR1 molecule was for the α_1 domain was reported by Klein and for the β_1 domain, the study reported by Todd et al. (Nature 329, 599 (1987)).

The sequence alignment is based on Brown et al. (Nature 332, 845 (1988)). The complete alignment and numbering scheme for both are seen in Figure 1. The Class II, β_1 and Class I α_2 domains regions were conserved with some variations at the ends where the two MHC's have different loop regions. The fourth B-strand in the α_1 domain of HLA-aw68 (residues 30-38) is disrupted in the DR1 model. Only three residues are in a β-sheet conformation, probably due to the inserted glycine at position 28 before the strand and the large deletion in the loop region immediately after the strand. The two alpha-helical regions are clearly maintained. Both helices have been observed to be discontinuous in the Class I molecules and are similar in the DR1 model. The α_1 domain helix is long and curves from residues 49α to 76α without significant disruption. It is essentially a single continuous helix. However, the α_2 helical region is broken into two separate helices as with the Class I molecules. A short helix (52-63) is separated from a longer helix (68-94) by a deformed region without secondary structure. This deformation is more pronounced in the DR1 model as opposed to the Class I molecules due to an insertion.

Influenza Hemagglutinin Peptide with DR1:

The amino acid residues 307-319 of influenza hemagglutinin (Pro-Lys- Tyr-Val-Lys-Gln-Asn-Thr-Leu-Lys-Leu-Ala-Thr, SEQ ID NO:1) make up a well-documented linear T-cell epitope which as been shown to be HLA-DR1 restricted. With the demonstration that the influenza hemagglutinin epitope (referred to as the HA peptide) binds DR1, it was chosen to be modeled into the binding cleft.

The peptide was initially inserted into the cleft so that Leu 11 HA was in the vicinity of the hydrophobic pocket. This allowed Asn 7 to be near the middle charged and polar groups of the cleft. The remaining residue of the motif (Lys 2) was near the vicinity of the remaining charged and polar residues at the end of the cleft. The only adjustment to the starting conformation was a slight rearrangement of the terminal peptide proline and Tyr 3 to alleviate obvious bad contacts.

After the energy minimization of the bimolecular complex, the total energy was reduced to 483 kcal/mol. This reduction in energy was accomplished by alleviation of several bad contacts and also by formation of several hydrogen bonds. The sticking feature of this mode is lack of hydrogen bonds in the carboxy terminal half of the peptide. Only one hydrogen bond is identified between the backbone carbonyl group of Leu 9 and the side chain of the β_1 Asn 77. In contrast, the amino terminal half has eleven identified interactions. Four of these interaction involve the peptide backbone residues Tyr 3, Val 4, and Gln 6. The remainder involve the side chains of Lys 2, Tyr 3, Lys 5 and Gln 6. Interestingly, Lys 5 is involved in more interactions

(three) than Lys 2 (only 2). No interactions were observed as anticipated with Asn 7. Instead it was the glutamine at position 6 donating a hydrogen bond to the α_1 Asn 62. No interactions were observed for the amino and carboxy termini.

HA-YK Peptide with DR1:

The binding of the HA-YK peptide (Ala-Ala-Tyr-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Lys-Ala-Ala, SEQ ID NO:2) to the DR1 model was tested. In aligning the peptide in the cleft, it was deemed logical to insert the tyrosine residue into the hydrophobic region of the binding cleft. The lysine would then be in position to interact with the hydrophilic groups in the other half of the cleft. The resulting peptide orientation is the opposite that used for the HA and the CS3 (defined below) peptides. With the peptide oriented as described, the final docking position for the peptide was unclear. The hydrophobic pocket is quite large, and, at least in this model, could accommodate the peptide tyrosine in a number of positions by sliding the peptide lengthwise through the cleft. However, repositioning the peptide also repositions the lysine. There were primarily two positions for the lysine: one with the lysine inside the cleft and the second with it outside. Of the two positions, the former was the lower in energy by 46 kcal/mol and had the greater number of interactions with the protein (11 vs. 7). Thus, the preferred orientation of the peptide appears to be with the lysine inside the binding cleft region.

CS3 subunit Pilin Peptide with DR1:

The suspected T-cell epitope for CS3 pilus subunit 63-78 (Ser-Lys-Asn-Gly-Thr-Val-Thr-Trp-Ala-His-Glu-Thr-

Asn-Asn-Ser-Ala, SEQ ID NO:3) was modeled with the DR1 molecule. The peptide was inserted with lysine inside the cleft in the hydrophilic region. This placed the Thr 5 in the center of the binding cleft and the tryptophane (residue 8) near the hydrophobic region. The resulting minimized model had ten interactions between the peptide and the protein, three interactions with the peptide backbone and five with the peptide side chains. The remaining two were with the amino terminal of the peptide. All of the interactions were in either the first three residues, His 10 or Glu 11 in the peptide. No interactions were observed in the center of the cleft or residues four through nine.

CFA/1 with DR1:

A peptide identified as CFA/1 (colonization factor antigen) (Val-Gly-Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Val-Asp-Pro, SEQ ID NO:4) was prepared and an attempt was made to "fit" the molecule into the cleft of the DR1. The lysine at position 3 prevented insertion of the peptide.

Results:

The peptides chosen to dock in the DR1 model are shown in Table 1. The peptides were docked manually in several orientations into the DR1 model. The peptides were then tested in biological binding assays with the following results:

Table I

Peptide	Molecular Model predicted binding	Binding in the bioassay
HA (influenza hemagglutinin)	Yes	Yes
HA-YK (synthetic peptide)	Yes	Yes
CS3 Pilin subunit	Yes	Yes
CFA/1	No	No

Quantitative measurement of the inhibition of CS3 63-78 and HA 306-318 as compared to controls is shown in Fig. 31.

The binding energy was calculated as the difference between the final DR1 and peptide complex and the sum of the energies for the minimized DR and peptide models individually. The data is shown in Table II.

Table II.

Peptide	Protein	Residues	Sequence	Binding Energy (kcal/mol)
HA	Influenza hemagglutinin	306-318	PKYVKQNTLKLAT, SEQ ID NO:1	-283
HA-YK	synthetic peptide		AAYAAAAAAKAA, SEQ ID NO:2	-216
CS3	CS3 pilin subunit	63-78	SKNGTVTWAHETNNSA,SEQ ID NO:3	-245

CS6α and CS6β with DR1

Colonization factor antigen IV (CFA/IV is an antigen on the surface of many entotoxigic *E. coli* one component of which is CS6. CS6 has two major subunits and a number of minor subunits. Several peptides from CS6 have been sequenced and assayed for potential inhibition of radiolabeled HA (306-318)/DR1 complex as a measure of immunogenicity. The sequences of the subunits are shown in Table III.

Table III.

Peptide	Amino Acid Residues	Sequence	
CS6α6	63-75	DEYGLGRLVNTAD,	SEQ ID NO:5
CS6α7	80-92	IIYQIVDEKGKKK,	SEQ ID NO:6
CS6α8	111-123	LNYTSGEKKISPG	SEQ ID NO:7
CS6ß1	3-15	WQYKSLDVNVNIE	SEQ ID NO:8
CS6ß2	42-54	QLYTVEMTIPAGV	SEQ ID NO:9
CS6ß3	112-124	TSYTFSAIYTGGE	SEQ ID NO:10
CS6ß4	123-135	GEYPNSGYSSGTY	SEQ ID NO:11
CS6ß5	133-145	GTYAGHLTVSFYS	SEQ ID NO:12

These peptides were assayed for inhibition of radioactivity labeled HA(306-318)/DR1. The results are demonstrated in Fig. 32.

The foregoing description of the specific embodiments reveal the general nature of the invention so that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation.

All references mentioned in this application are incorporated by reference.